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## Direct Demonstration of ATP-dependent Release of SecA from a Translocating Preprotein by Surface Plasmon Resonance\*

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**Translocase mediates the transport of preproteins across the inner membrane of *Escherichia coli*. SecA binds with high affinity to the membrane-embedded protein-conducting SecYEG complex and serves as both a receptor for secretory proteins and as an ATP-driven molecular motor. Cycles of ATP binding and hydrolysis by SecA drive the progressive movement of the preprotein across the membrane. Surface plasmon resonance allows an online monitoring of protein interactions. Here we report on the kinetic analysis of the interaction between SecA and the membrane-embedded SecYEG complex. Immobilization of membrane vesicles containing overproduced SecYEG on the Biacore Pioneer L1 chip allows the detection of high affinity SecA binding to the SecYEG complex and online monitoring of the translocation of the secretory protein proOmpA. SecA binds tightly to the SecYEG-proOmpA complex and is released only upon ATP hydrolysis. The results provide direct evidence for a model in which SecA cycles at the SecYEG complex during translocation.**

SecA (1) is the central component of the translocase, a multimeric enzyme complex that mediates the translocation of secretory proteins (preproteins) across the inner membrane of *Escherichia coli* (reviewed in Ref. 2). SecA is a large (102 kDa) homodimeric protein (3, 4) that localizes in the cytosol and associates peripherally with the inner membrane. At the membrane, SecA functions as a preprotein receptor and as an ATP-driven molecular motor that threads the preprotein through a pore formed by an oligomeric assembly of the integral SecYEG membrane protein complex (5–8). SecA binds the SecYEG complex with high affinity. This interaction occurs at least via direct association with SecY (9). SecG is not required for high affinity SecA binding (10), but cross-linking studies indicate that both proteins interact physically during the SecA reaction cycle (11). In addition, SecA associates with low affinity with phospholipids (12).

SecA contains two nucleotide binding domains (NBDs)<sup>1</sup> that

are both indispensable for protein translocation (13, 14). NBD1 is a high affinity catalytic NBD ( $K_D$  ADP = 0.15  $\mu$ M) located near the N terminus of the protein. This domain shares significant structural similarity with the DEAD motor domains of helicases (15). The more distally located NBD2 may have a regulatory function instead of being a true ATP hydrolytic site (16, 17). The events that take place during ATP-driven protein translocation have been partially resolved. SecYEG-bound SecA binds preproteins via direct recognition of the signal sequence and mature domain (12, 18). Translocation of the preprotein is driven by multiple cycles of ATP binding and hydrolysis at SecA (19, 20). Each catalytic cycle of SecA involves two distinct translocation steps that utilize the energy of either preprotein or ATP binding to allow the translocation of the preprotein in 20- to 30-amino acid steps (19). Although several studies have addressed the order of events during protein translocation, relatively little is known about the SecA-SecYEG interaction in this process. SecA has been proposed to partition between the cytoplasm and the inner membrane during its reaction cycle (19, 21), but other studies question the SecA membrane cycling model as a significant fraction of active SecA remains stably associated with the membrane throughout the translocation reaction (22). Indirect evidence, using SecA bound to a liposome surface instead to SecYEG, suggests that ATP hydrolysis drives the release of the SecA-bound preprotein (19). To address the SecA cycling model directly, we have studied the SecA-SecYEG interaction by surface plasmon resonance (SPR). SPR is a versatile technique that allows rapid, online monitoring of protein interactions. We have used the Biacore Pioneer L1 chip to immobilize inner membrane vesicles (IMVs) containing wild-type and overproduced levels of SecYEG. The immobilized vesicles bind SecA with high affinity in a SecYEG-dependent manner and could be used to monitor protein translocation online. The data demonstrate that SecA binds tightly to the SecYEG complex when it contains a trapped preprotein translocation intermediate. Dissociation from the SecYEG-preprotein complex requires ATP hydrolysis. The results provide direct evidence for a translocation model in which SecA can cycle between a soluble and SecYEG-associated state in an ATP-dependent manner.

### EXPERIMENTAL PROCEDURES

**Materials**—*E. coli* SecA (1), SecB (23), proOmpA (24), and Cys-less proOmpA (25) were purified as described. IMVs containing wild-type levels of SecYEG, overexpressed His-tagged SecYEG, or overexpressed His-tagged SecYE were obtained from *E. coli* SF100 cells transformed with pET302 (26), pET610 (27), or pET320 (28) as described (27). ADP, ATP, AMP-PNP, and bovine serum albumin were from Sigma.

**SPR**—SPR measurements were performed using a two-channel BIAlite SPR system (Biacore AB, Uppsala, Sweden). IMVs (2 mg/ml in Buffer A (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol) were passed 11 times through a 100-nm polycarbonate membrane (Avestin, Ottawa, Ontario, Canada) and immobilized on a Pioneer L1 sensor chip (Biacore AB, Uppsala, Sweden). Binding exper-

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<sup>1</sup> The abbreviations used are: NBD, nucleotide binding domain; AMP-PNP, adenosine 5'-( $\beta$ , $\gamma$ -imino)triphosphate; SPR, surface plasmon resonance; IMV, inner membrane vesicle; RU, response units; WT, wild-type.

iments were performed in Buffer A with 0.5 mg/ml bovine serum albumin and 150 mM KCl at 25 or 30 °C. Stability of the baseline and regeneration of SecA binding sites were obtained by injection of 100 mM Na<sub>2</sub>CO<sub>3</sub>, pH 10.

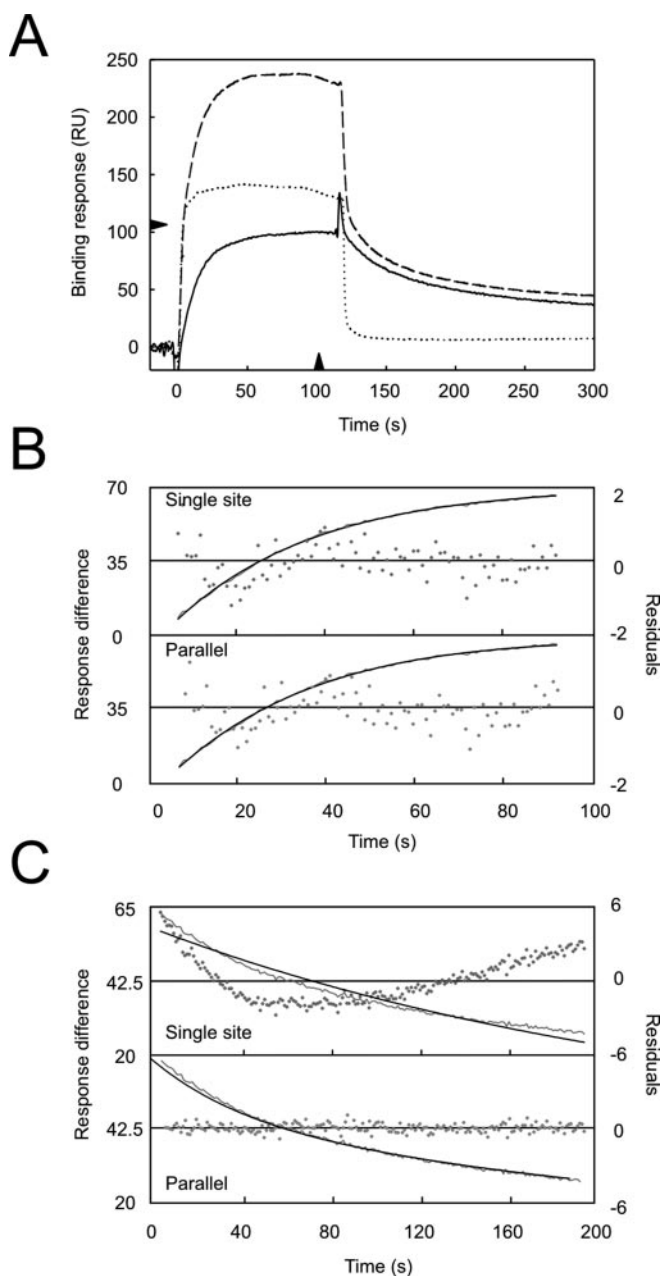
**Data Analysis**—The SPR response prior to injection and the time of injection were adjusted to zero. Binding curves were corrected for changes in the refractive index of the buffer and nonspecific binding to IMVs and the chips surface by the BiacoreX control software 2.0.1 (Biacore AB, Uppsala, Sweden). Corrected binding curves of SecA association and dissociation were quantitatively analyzed using the BIAevaluation 2.2.4 software (Biacore AB, Uppsala, Sweden). The data were fitted to a pseudo-first order model of simple 1:1 binding ( $A + B \leftrightarrow AB$ ) by non-linear regression. The dissociation rate constant was derived from the equation  $R_t = R_0 e^{-k_{\text{off}}(t-t_0)}$  where  $R_t$  is the response in response units (RU) at time  $t$ ,  $R_0$  is the response at  $t_0$ , and  $k_{\text{off}}$  is the dissociation rate constant. The association rate constant  $k_{\text{on}}$  was derived from the equation  $R_t = R_{\text{eq}}(1 - e^{-(k_{\text{on}}[C] + k_{\text{off}})(t-t_0)})$ , where  $[C]$  is the ligand concentration, and  $R_{\text{eq}}$  is the steady state response level. In addition, the dissociation phase was fitted to a parallel dissociation model ( $AB_1 + AB_2 \leftrightarrow A + B_1 + B_2$ ), using the equation  $R_t = R_1 e^{-k_{\text{off1}}(t-t_0)} + (R_0 - R_1) e^{-k_{\text{off2}}(t-t_0)}$ . The affinity ( $K_D$ ) was calculated from the ratio  $k_{\text{off}}/k_{\text{on}}$ , and from the response levels attained at equilibrium for different SecA concentrations by Scatchard analysis.

**Other Biochemical Techniques**—Protein concentrations were determined using the Bio-Rad DC protein assay with bovine serum albumin as a standard.

## RESULTS

**Detection of SecA-SecYEG Interaction by Surface Plasmon Resonance**—To study the interaction between SecA and the SecYEG complex, IMVs containing overexpressed SecYEG (SecYEG<sup>+</sup>) were immobilized on a Pioneer L1 sensor chip. The surface of this sensor chip consists of dextran modified with lipophilic anchoring molecules that protrude the lipid bilayer thereby immobilizing the vesicles. The Bialite SPR system contains two flow cells that enable simultaneous monitoring of a sample and a control. A control surface was prepared by loading IMVs containing wild-type (WT) levels of SecYEG onto the second cell. IMV immobilization caused an SPR response between 2500 and 4500 RU (data not shown). Loading levels of the control (WT) and sample (SecYEG<sup>+</sup>) cells were similar to allow direct comparison.

The binding of SecA to the immobilized IMVs was followed in time (Fig. 1A). The responses of the WT and SecYEG<sup>+</sup> IMVs in Fig. 1A include a “bulk” contribution to the SPR signal, *i.e.* a change in the signal because of changes in the refractive index of the injection buffer. After bulk correction, the SecYEG<sup>+</sup> response was increased five times compared with the WT response, demonstrating specific SecA-SecYEG interaction. Specific binding of SecA to SecYEG reached saturation of available binding sites at a SecA concentration of about 48 nM (data not shown). The association and dissociation rates of the SecA-SecYEG interaction were determined after correction of the SecYEG<sup>+</sup> IMV response for the WT IMVs response, which includes the bulk contribution and nonspecific SecA binding (see Fig. 1A and Table I). The association of SecA with the SecYEG complex fitted best to a pseudo-first order model of simple 1:1 binding ( $A + B \leftrightarrow AB$ ), with a  $k_{\text{on}}$  of  $2.5 \pm 0.4 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$  (see Fig. 1B and Table I). The dissociation phase was more accurately described by a parallel dissociation model ( $AB_1 + AB_2 \leftrightarrow A + B_1 + B_2$ ) with a  $k_{\text{off1}}$  of  $2.8 \pm 0.4 \times 10^{-2} \text{ s}^{-1}$  (27%) and a  $k_{\text{off2}}$  of  $3.9 \pm 0.4 \times 10^{-3} \text{ s}^{-1}$  (73%) (see Fig. 1C and Table I). Parallel dissociation can be caused by the presence of two ligand binding sites with different affinities. However, this phenomenon has been reported to occur frequently in SPR experiments and can also be attributed to steric hindrance (molecular crowding), mass transport limitations, or non-uniform immobilization of the binding sites (29). The  $k_{\text{off1}}$  of the parallel dissociation model yielded an unrealistic (negative)  $k_{\text{on}}$ ; therefore, the parallel character of the dissociation phase probably does not reflect the existence of two different



**FIG. 1. Specific SecA-SecYEG interaction visualized by SPR.** A, SPR sensogram of the binding of SecA (48 nM) to a Pioneer L1 sensor chip covered with IMVs containing wild-type (dotted line) or overexpression levels of SecYEG (dashed line). The solid line indicates the corrected, specific SecA binding. The arrowheads on the x and y axes indicate the end of the injection and the contribution of the bulk flow, respectively. Binding experiments were performed at a flow rate of 20  $\mu\text{L}/\text{min}$  at 25 °C. A small fraction of SecA was slowly released. B, fit of the association phase of specific SecA binding to IMVs containing overexpressed SecYEG. The data (gray lines) were fitted (black lines) to a model assuming homogeneous single-site interaction ( $A + B \leftrightarrow AB$ ) or a model assuming parallel interaction ( $A + B_1 + B_2 \leftrightarrow AB_1 + AB_2$ ). C, fit of the dissociation phase of specific SecA binding to IMVs containing overexpressed SecYEG. Data (gray lines) were fitted (black lines) to a model assuming homogeneous single-site or parallel interaction.

SecA binding sites. The affinity ( $K_D$ ) determined by the ratio  $k_{\text{off}}/k_{\text{on}}$  (Table I) was comparable with the SecA binding affinity reported in previous studies (30–32). The kinetic parameters for SecA binding kinetics were independent of SecA concentration (3–96 nM), and an increased flow rate from 20 to 40  $\mu\text{L}/\text{min}$  yielded comparable response curves, indicating that the kinetics were not influenced by mass transport limitations. To validate the kinetic analysis of the SecA-SecYEG interaction, the



TABLE I  
Kinetic constants of SecA-SecYEG interaction

IMVs	$k_{\text{on}}$ $M^{-1} s^{-1}$	$k_{\text{off}}$ $s^{-1}$	$k_{\text{off1}}$ $s^{-1}$	$k_{\text{off2}}$ $s^{-1}$	$K_D$ $k_{\text{off}}/k_{\text{on}}$ $M$
SecYEG	$2.5 \pm 0.4 \times 10^6$	$5.2 \pm 0.4 \times 10^{-3}$	$2.8 \pm 0.4 \times 10^{-2}$ (27%)	$3.9 \pm 0.4 \times 10^{-3}$ (73%)	$2.0 \pm 0.2 \times 10^{-9}$
SecYE	$3.1 \pm 0.4 \times 10^6$	$5.3 \pm 0.4 \times 10^{-3}$	$2.6 \pm 0.4 \times 10^{-2}$ (26%)	$4.2 \pm 0.4 \times 10^{-3}$ (74%)	$1.9 \pm 0.1 \times 10^{-9}$

results were subjected to the self-consistency tests proposed by Schuck and Minton (33). The  $K_D$  determined by Scatchard analysis ( $3.6 \pm 0.2$  nM for SecYEG) from the response levels attained at equilibrium for different SecA concentrations was comparable with the  $k_{\text{off}}/k_{\text{on}}$  ratio. The  $k_{\text{off}}$  calculated using linear regression of the dependence of  $k_{\text{obs}}$  on  $[C]$  ( $k_{\text{obs}} = k_{\text{on}}[C] + k_{\text{off}}$ , where  $[C]$  is the SecA concentration) was approximately equal to  $k_{\text{off}}$  determined by fitting to  $R_t = R_0 e^{-k_{\text{off}}(t - t_0)}$  and the  $k_{\text{obs}} > k_{\text{off}}$  for all SecA concentrations tested (data not shown). SPR experiments with immobilized vesicles containing SecYE instead of SecYEG (Table I) yielded identical results. This confirms a previous study (10) in which SecG was shown not to contribute to the high affinity binding of SecA to the SecYE complex. Taken together, these data demonstrate that SPR can be used to directly monitor the kinetics of the high affinity interaction between SecA and the SecYEG complex.

**Binding of proOmpA to the SecA-SecYEG Complex**—SecYEG-bound SecA binds preproteins either in a complex with the chaperone SecB or as free preprotein, but the exact affinity of the interaction of SecA with preproteins is not known (34). To characterize the interaction of the preprotein proOmpA with SecYEG-bound SecA in more detail by SPR, IMVs were immobilized as described above and saturated with SecA. Subsequently, proOmpA was injected in either the presence or absence of SecB. In the absence of SecB, proOmpA binding resulted in a response of 60 RU (Fig. 2, *dashed line*) and occurred with an affinity of  $14 \pm 1$  nM (Table II). In the presence of SecB the binding response increased to 120 RU (Fig. 2, *solid line*). This increased response is probably because of the binding of the larger proOmpA-SecB complex, because binding of SecB alone could not be detected under the conditions applied (Fig. 2, *dotted line*). Biochemical analysis demonstrated that the SecB was normally active (data not shown). The proOmpA-SecB complex bound to the SecA-SecYEG complex with an affinity of  $16 \pm 2$  nM (Table II), which is nearly identical to the affinity of proOmpA binding in the absence of SecB.

**Protein Translocation Monitored by SPR**—To determine whether SPR can be used to monitor the interactions that occur during the protein translocation cycle, experiments were conducted in the presence of ATP. Injection of SecA, proOmpA, and SecB resulted in an SPR response of  $\sim 200$  RU (Fig. 3A, *dashed line*), which corresponds largely to the sum of the individual binding steps of SecA (100 RU; see Fig. 1) and the proOmpA-SecB complex (120 RU; see Fig. 2). When ATP was included in the injection buffer, a significant increase in the SPR response was observed that did not reach saturation at the end of the association phase (Fig. 3A, *solid line*). To address the possibility that the increased SPR response with time was caused by translocation of injected proOmpA, an experiment was conducted that allowed for only one round of translocation. Oxidation of proOmpA induces the formation of an internal disulfide bridge between Cys<sup>290</sup> and Cys<sup>302</sup>, which causes a translocation arrest in the absence of the proton motive force (19, 35). As a consequence, translocated proOmpA accumulates as a translocation intermediate ( $I^{29}$ ) that occupies the translocation site and blocks it for a second round of translocation (7, 19, 35). The alkaline carbonate treatment after the immobilization of the vesicles removes the  $F_1$  domain of the  $F_0F_1$ -ATPase and thus the ability to generate a proton motive force

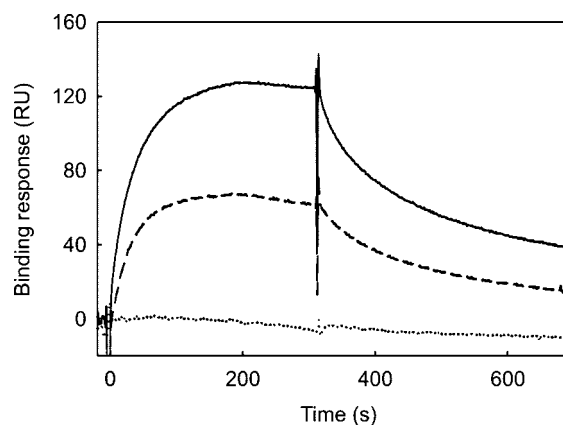


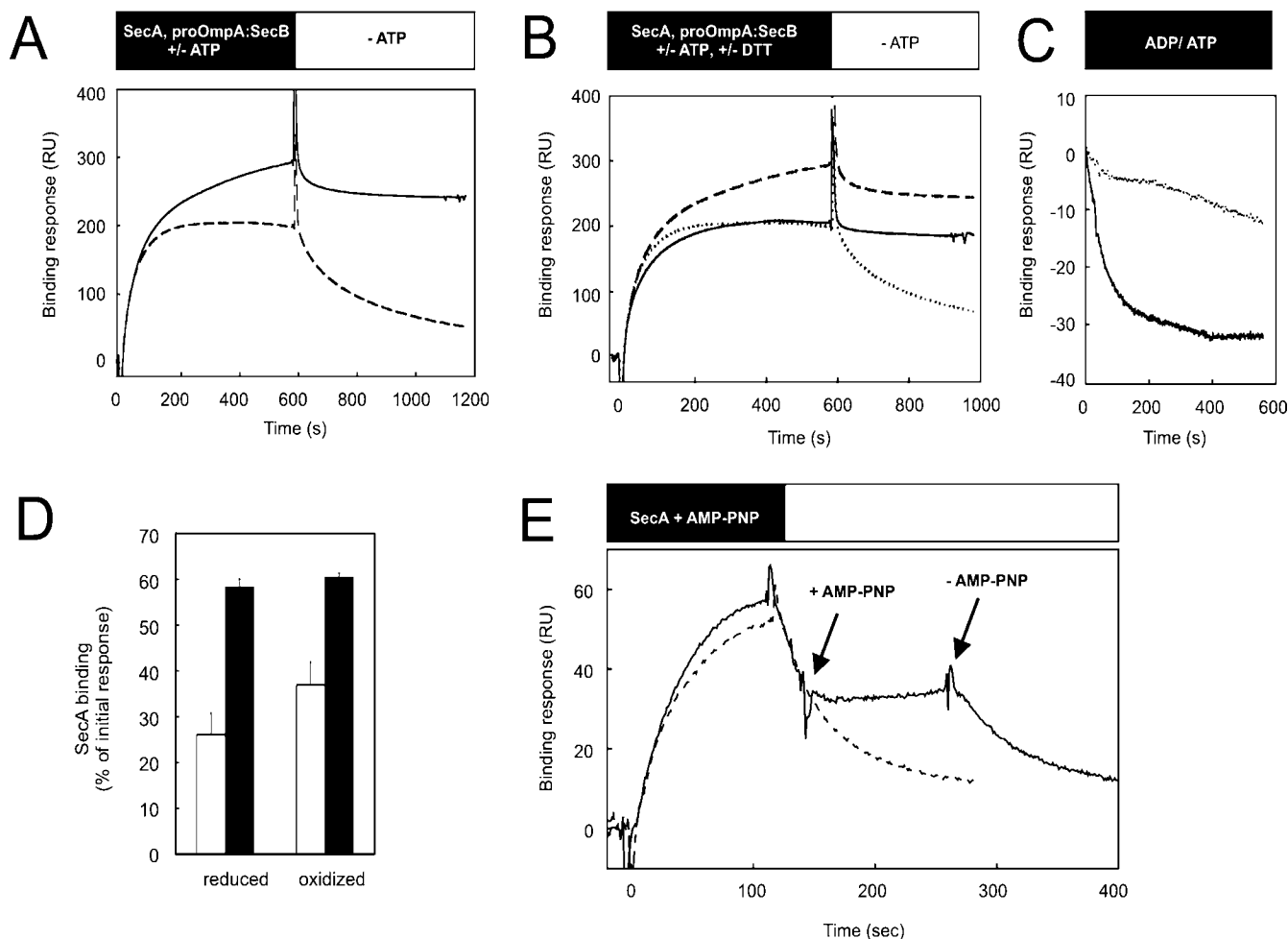
FIG. 2. **Interaction of proOmpA with the SecA-SecYEG complex.** IMVs were immobilized on the L1 chip as described and saturated with SecA using a running buffer containing 48 nM SecA. proOmpA (100 nM; *dashed line*), SecB (100 nM; *dotted line*) or a proOmpA:SecB (*solid line*) complex were injected at a flow rate of 20  $\mu$ l/min. Binding experiments were done at 25 °C. The corrected data representing the specific binding to the SecA-SecYEG complex are shown.

TABLE II  
Kinetic constants of the interaction of proOmpA or proOmpA:SecB with SecYEG bound SecA

Ligand	$k_{\text{on}}$ $M^{-1} s^{-1}$	$k_{\text{off}}$ $s^{-1}$	$K_D$ $M$
proOmpA	$2.6 \pm 0.1 \times 10^5$	$3.6 \pm 0.1 \times 10^{-3}$	$14 \times 10^{-9}$
proOmpA:SecB	$1.9 \pm 0.3 \times 10^5$	$2.9 \pm 0.1 \times 10^{-3}$	$16 \times 10^{-9}$

upon the addition of ATP (data not shown). When SecA, proOmpA-SecB, and ATP were injected under oxidizing conditions, the SPR signal saturated quickly and reached a much lower level than under reducing conditions (Fig. 3B). The maximal response for the injection of SecA, ATP, and oxidized proOmpA-SecB ( $\sim 200$  RU; see Fig. 3B, *solid line*) was comparable with the response of an injection of SecA and proOmpA-SecB without nucleotides (Fig. 3B, *dotted line*). This is consistent with the observation that oxidized proOmpA blocks the translocation sites for further translocation (7, 19, 35). Oxidation of a Cys-less proOmpA mutant yielded a response similar to wild-type proOmpA under reducing conditions (data not shown). After reduction of the disulfide bond with dithiothreitol, addition of SecA, proOmpA-SecB, and ATP resulted in a further increase in the SPR response consistent with a new cycle of translocation (data not shown). These results demonstrate that the immobilized vesicles are capable of translocation and that SPR can be used to monitor protein-protein interactions during translocation.

**Release of SecA from a SecYEG-trapped Preprotein Is ATP-dependent and Does Not Require Completion of Translocation**—In the absence of ATP, binding of SecA and proOmpA-SecB was reversible, and dissociation occurred immediately at the end of the injection (Fig. 3A, *dashed line*). In contrast, when the complex was formed under translocation conditions (*i.e.* with ATP), it remained stably associated with the membrane (Fig. 3A, *solid line*) and was even resistant against alkaline carbonate treatment (data not shown). This

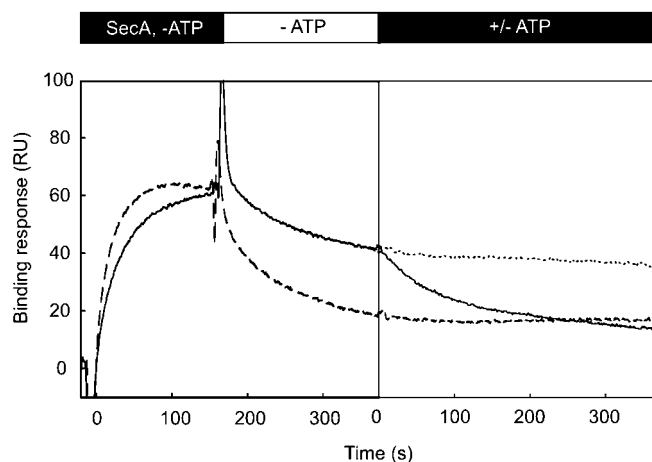


**FIG. 3. Binding of a SecA-proOmpA complex to SecYEG.** *A*, binding of SecA (48 nM), proOmpA (100 nM), and SecB (100 nM) to IMVs containing overexpressed SecYEG in the presence (black line) or absence (dashed line) of ATP (1 mM). The buffer compositions during the association and dissociation phase are indicated in the black and white bars, respectively. *B*, binding of SecA (48 nM) and proOmpA:SecB to SecYEG<sup>+</sup> IMVs under oxidizing (solid line) or reducing conditions in the presence (dashed line) or absence (dotted line) of ATP (1 mM). The buffer compositions during the association and dissociation phase are indicated in the black and white bars, respectively. *C*, injection of 1 mM ATP (solid line) or ADP (dotted line) over a complex formed in the presence of ATP (see *A*). *D*, response of a second round of SecA binding before (white bars) or after injection of ATP (black bars) over the SecA-proOmpA-SecB complex formed in the presence of ATP under reducing or oxidizing conditions (see *B*). Binding responses were compared with the binding response of SecA in the absence of translocation ligands (initial response) (Fig. 3D). When complex dissociation was analyzed in the absence of ATP, a second SecA injection resulted in a response of only 30% of the initial response (Fig. 3D). However, when the complex was allowed to dissociate in the presence of ATP, a second injection of SecA resulted in a response of 55% (Fig. 3D). The increase in SPR response corresponds to the amount of material that dissociated from the membrane upon injection of ATP (Fig. 3C). This suggests that

can be explained in part by translocation of proOmpA, but this reaction is not expected to inhibit dissociation of SecA. Only upon a second injection with ATP, part of the complex dissociated from the membrane, whereas ADP was unable to elicit the release (Fig. 3C). To test whether this dissociation represented SecA that was associated previously with the SecYEG complex, the recovery of high affinity SecA binding sites was monitored by a new injection with SecA. For this purpose, SecA was first injected together with the translocation ligands proOmpA, SecB, and ATP and allowed to dissociate in the presence or absence of ATP. Subsequently, a second injection of SecA was passed over the sensor chip, and the binding responses were compared with that of SecA in the absence of translocation ligands (initial response) (Fig. 3D). When complex dissociation was analyzed in the absence of ATP, a second SecA injection resulted in a response of only 30% of the initial response (Fig. 3D). However, when the complex was allowed to dissociate in the presence of ATP, a second injection of SecA resulted in a response of 55% (Fig. 3D). The increase in SPR response corresponds to the amount of material that dissociated from the membrane upon injection of ATP (Fig. 3C). This suggests that

ATP is able to release part of the complex from high affinity SecA binding sites. ADP or the non-hydrolysable ATP analogue AMP-PNP failed to dissociate the complex, and under those conditions no high affinity binding sites for SecA were recovered (data not shown). These results demonstrate that the dissociation of SecA from the membrane after one or more rounds of translocation requires the hydrolysis of ATP. An ATP-dependent release from the membrane has previously been proposed based on the behavior of a protease-protected 30-kDa fragment of SecA (21, 36). In these studies, formation of the 30-kDa fragment could also be driven by AMP-PNP in the absence of a preprotein. SPR showed that SecA dissociated readily after it had been bound to the SecYEG complex in the presence of AMP-PNP (Fig. 3E, dotted line). The dissociation could, however, be blocked by a second injection with AMP-PNP (Fig. 3E, solid line). These results show that also a non-hydrolyzable ATP analogue can lock SecA at the membrane, but in contrast to the complex formed in the presence of preprotein and ATP, the AMP-PNP-dependent complex readily dissociates upon depletion of the nucleotide.

The ATP injection that is required for the dissociation of



**FIG. 4. SecA binding to a proOmpA-SecYEG complex.** The translocation complex was formed under oxidizing (solid line) or reducing (dashed line) conditions (see Fig. 3B), and SecA was dissociated from the translocation complex by an injection with an ATP-containing buffer. *Left panel*, rebinding of SecA after formation of a translocation complex. *Right panel*, dissociation of rebound SecA from the oxidized complex in the presence (solid line) or absence (dotted line) of ATP or from the reduced complex in the absence of ATP (dashed line). All binding experiments were performed at 30 °C. SecA, proOmpA, SecB, and ATP were present at a concentration of 48 nM, 100 nM, 100 nM, and 1 mM, respectively. The corrected data representing the specific binding to the proOmpA-SecYEG complex are shown.

SecA could be necessary to complete the translocation of the partially translocated preproteins. Alternatively, SecA could dissociate from the membrane upon ATP hydrolysis irrespective of completion of translocation. To discriminate between these possibilities, the dissociation of SecA from the oxidized proOmpA translocation intermediate was studied. Similar to the complex formed under reducing conditions, the oxidized complex did not dissociate from the membrane at the end of injection (Fig. 3B). Dissociation and regeneration of SecA binding sites could be obtained by addition of ATP (Fig. 3D), implying that completion of translocation is not required for the release of SecA from the translocation site. To analyze the re-association of SecA with translocation sites that are occupied by partially translocated preproteins, binding of SecA to an oxidized proOmpA-SecYEG complex was examined. SecA was injected together with the translocation ligands proOmpA-SecB and ATP under either oxidizing or reducing conditions. After the end of the injection, complexes were allowed to dissociate in the presence of ATP. Subsequently, a second injection of SecA was passed over the sensor chip, and the binding responses were compared. After preincubation under oxidizing or reducing conditions, the second SecA injection resulted in a similar SPR response of ~60 RU (see Fig. 3D and Fig. 4, *left panel*). The dissociation phase, however, was different for the two conditions. When SecA was bound to the oxidized proOmpA-SecYEG complex it remained stably associated with the membrane (Fig. 4, *left panel*). Again ATP was required for dissociation (Fig. 4, *right panel*), whereas AMP-PNP and ADP were unable to release SecA.

The second round of SecA binding to the complex formed under reducing conditions was reversible, and dissociation occurred immediately after the end of the injection (Fig. 4, *left panel*). In contrast to oxidized proOmpA, reduced proOmpA does not form stable translocation intermediates. Likewise, after reduction of the disulfide bond of oxidized proOmpA with dithiothreitol, a second injection of SecA also resulted in reversible binding (data not shown). Conversion of the intermediate I<sup>29</sup> to full-length translocated proOmpA can be driven by low ATP concentrations once the disulfide bridge is reduced

(19). Taken together, these results show that SecA binds with a very high affinity to the SecYEG complex when it contains a partially translocated preprotein and that effective dissociation of this preprotein-SecYEG complex requires the hydrolysis of ATP.

## DISCUSSION

The SecA motor of the *E. coli* preprotein translocase is a highly dynamic protein that in the cell distributes between cytosolic and membrane-associated states (37). The interaction of SecA with the inner membrane has been studied previously by binding experiments using radiolabeled SecA. This demonstrated that the SecA association with the membrane occurs with high affinity with the SecYEG complex (34) and with low affinity with the negatively charged lipids (12). Unfortunately, this discontinuous binding assay provides no information about the kinetics of the interaction and cannot easily be applied when protein translocation conditions prevail. To directly monitor the SecA interaction with SecYEG, we have analyzed this process by SPR. This technique has been used extensively for studies on soluble proteins, but the method has only recently become applicable for studies on the interaction with membranes. So far, these studies only concerned the interaction between a soluble protein or peptide and a lipid bilayer. The results presented here show that SPR can be used to obtain detailed and quantitative information on the interaction between the peripheral SecA protein and its membrane receptor, the SecYEG complex. The affinity for the binding of SecA to SecYEG determined by SPR is comparable with values determined previously (30) by binding experiments with radiolabeled SecA. In addition, SPR could be used to study the interactions that occur during protein translocation.

Based on the formation of a protease-protected 30-kDa fragment, the SecA reaction cycle has been proposed to involve nucleotide-induced membrane insertion and de-insertion steps (21). Because the 30-kDa fragment could be chased by an excess of non-radioactive SecA, SecA was proposed to alternate between a cytosolic and membrane-associated state during its reaction cycle (21). However, other studies failed to demonstrate a translocation-dependent dissociation of membrane-bound SecA and concluded that membrane cycling is not an essential feature for protein translocation (22). The SPR results presented here demonstrate directly that SecA can dissociate from the activated SecYEG complex during a translocation cycle. In the absence of a preprotein, SecA binding to the SecYEG complex is readily reversible. However, when SecA is actively engaged with a preprotein trapped in the translocation channel, SecA release strictly requires the hydrolysis of ATP. The ATP-dependent dissociation is not related to completion of translocation, because SecA also dissociates in an ATP-dependent manner from partially translocated preproteins that remain trapped because of the presence of disulfide bridge. These data confirm previous suggestions that upon ATP hydrolysis, SecA releases the bound preprotein (19). However, in contrast to our SPR study, this conclusion was based on an experiment with SecA bound to lipid vesicles in the absence of the SecYEG complex under non-translocating conditions.

The SPR observations also show resemblance to the behavior of the SecA proteolytic 30-kDa fragment that can be chased by an excess of non-radioactive SecA in the presence of ATP (21), although this experiment does not demonstrate net release of SecA from the translocation site. In the absence of a preprotein, the 30-kDa fragment of SecA could be formed in the presence of non-hydrolyzable ATP analogs, and formation of the 30-kDa SecA fragment was attributed to the binding of ATP during the translocation reaction (36). The requirements for the formation of a stable SecA-SecYEG complex that was detected by SPR are



different. Various reports suggest that the 30-kDa fragment formed in the presence of AMP-PNP may differ from the one formed under translocation conditions (36, 39). The SPR experiments demonstrate that SecA readily dissociated from the SecYEG complex after injection in the presence of AMP-PNP, unless the nucleotide was also present during the dissociation. A SecA-SecYEG complex that remained stable after depletion of nucleotides could only be formed under translocation conditions or when SecA re-bound to the SecYEG complex containing a partially translocated preprotein. This suggests that stable membrane association of SecA under translocation conditions is dependent on the presence of a preprotein rather than the binding of ATP. The presence of the preproteins is, however, not sufficient as a dissociable SecA-preprotein-SecYEG complex is formed when SecA and proOmpA are injected in the absence of ATP (Fig. 3, A and C). Apparently, stable membrane association of SecA does not result from the binding of ATP directly but needs to be preceded by an ATP-dependent preprotein translocation step. A possible explanation for this phenomenon is that SecA interacts with high affinity with the exposed non-translocated polypeptide domains of the preprotein. This may anchor SecA more stable to the SecYEG pore complex such that ATP hydrolysis is needed to dissociate the SecA-preprotein interaction (19). Alternative explanations need to be considered, as well. SecA has been proposed to be subjected to changes in its quaternary structure during its reaction cycle (40–42). Differences in the oligomeric state of SecA could affect the affinity of the SecA-SecYEG interaction, but in our SPR experiments we cannot discriminate between a monomeric and dimeric state of SecA at the translocase. Because of the presence of a trapped translocation intermediate, the SecYEG complex may exist in a different conformational state that permits a tight interaction with SecA. Biochemical and electron microscopy studies have indicated that the translocation pore is formed by an oligomeric assembly of SecYEG complexes (7). In a three-dimensional structure reconstituted from two-dimensional SecYEG crystals the SecYEG complex is dimeric and displays a deep cavity at the dimer interface (38). Because this cavity is closed at the periplasmic side of the membrane, the structure was proposed to represent a “closed state” of the translocation channel (38). The structure would convert into an “open state” when the complex is actively involved in translocation, but it is not clear whether the open state reflects a different conformational or oligomeric state. In this respect, electron microscopy suggests that SecYEG dimers tetramerize upon activation of SecA by AMP-PNP or ATP and a preprotein (7). Although the oligomeric state of the active SecYEG pore complex remains a topic of debate, both models propose that the protein-conducting pore needs to be activated at the initiation of translocation. The ATP-dependent step that is required for the stable membrane association of SecA could be related to this activation of the translocation pore. In such a scenario, SecA binds to the inactive SecYEG complex in a reversible manner. Upon binding of a preprotein and ATP to SecYEG-bound SecA the SecYEG complex is activated, and this event locks SecA at the translocation site. When SecA is released from the membrane after the hydrolysis of ATP, the trapped translocation intermediate can stabilize the pore in the activated state. As a consequence, in a second reaction cycle SecA can bind tightly to the SecYEG complex in the absence of ATP. In a non-arrested reaction cycle, re-binding of SecA to the translocation intermediate results in a translocation progress (19, 20).

In conclusion, this study demonstrates for the first time that SPR can be used to monitor the association and dissociation

kinetics of a peripheral protein component with its membrane receptor embedded in its natural lipid environment. The SPR results confirm a translocation model in which SecA alternates between a free (cytosolic) and membrane-associated state. The study further demonstrates that during the translocation reaction, SecA is tightly bound to the activated SecYEG complex but can be released upon the hydrolysis of ATP.

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